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(54) **Sterilization indicator using DNA specific dyes**

DNS-spezifische Farbstoff verwendender Sterilisationsindikator

Indicateur de stérilisation utilisant les colorants spécifiques à l'ADN

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- **PANASCI L ET AL: "THE EFFECT OF PROLONGED INCUBATIONS AND HEAT DENATURATION ON MELPHALAN-INDUCED DNA CROSS-LINKS AS MEASURED BY THE ETHIDIUM BROMIDE FLUORESCENCE ASSAY" CANCER LETTERS, vol. 50, no. 2, 1990, pages 129-132, XP008001462 ISSN: 0304-3835**



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## Description

**[0001]** The present Invention is directed to an improved sterilization indicator, particularly one inserted into the sterilization chamber and based upon the interaction between DNA and dyes.

## BACKGROUND OF THE INVENTION

**[0002]** Both hospitals and doctors' offices customarily require the presence of devices for sterilization of various medical instruments. These sterilizers are of two basic types, i.e. prevacuum and gravity. In the former case, air is evacuated from the sterilization chamber before the sterilization cycle is initiated. This assists the steam generated in penetrating both the sterilization indicator and the instruments being treated. The gravity cycle is similar to the prevacuum cycle except that no vacuum is applied. The former is almost universally used in hospitals and the latter in doctors' offices and small clinics. Overall, approximately 25% of all sterilizers are of the gravity type, while the remaining are prevacuum. Since the gravity type is smaller and more compact, establishments such as individual doctors' offices and small clinics tend toward their use. The prevacuum type is larger and cumbersome, and is capable of being operated as both a prevacuum sterilizer and a gravity sterilizer.

**[0003]** Therefore, it is of importance that any sterilization indication method be suitable for both types of sterilizers.

## SUMMARY OF THE INVENTION

**[0004]** The present invention is defined by the claims.

**[0005]** More specifically, the invention method of determining the effectiveness of the sterilization cycle includes placing the sterilization indicator in the sterilization chamber prior to beginning the sterilization cycle. The indicator contains a DNA preparation consisting of 0.25 to 1.0 μg of initial DNA per μl of water. A preferred concentration is 0.25 to 0.50 μg of initial DNA per μl. After the sterilization cycle has been completed, the DNA is withdrawn from the sterilization chamber and placed on a support. Preferably, the support is a plastic having a negative surface potential, especially nylon or a nitrocellulose membrane. The latter two have a particular affinity for the DNA. The dye solution is placed on the DNA and thereafter dipped into water and any change in color is observed.

**[0006]** The DNA has the ability to bind dyes within its structure, provided that the molecules are whole and complete. However, when the DNA is subjected to a combination of heat and steam (heat alone is insufficient), the structure breaks down so that the binding is less efficient. Thus, if sufficient heat and steam has contacted the DNA during the sterilization cycle, it will be fragmented and will not retain the dye. Therefore, when the membrane (for example) is dipped into the dye solution and then into water, a substantial and easily observable portion of the dye is dissolved. What remains on the paper looks substantially different from the original.

**[0007]** On the other hand, if the sterilization cycle is not complete, then the DNA molecule is substantially intact. As a result, the dye remains bound and very little will be washed off when it is dipped. Thus, the person carrying out the test can easily determine whether the cycle has done its job properly. Moreover, the results of the test are immediate; there is no need to wait for days or even hours to determine whether a given sterilization cycle has been suitably effective.

**[0008]** The DNA used is high molecular weight double stranded DNA. Obtaining the DNA from salmon sperm is the most economical way of providing this substance, but the source is not critical.

**[0009]** As to the dyes, ethidium bromide is the most tightly bound, followed by methylene blue. The former, however, is carcinogenic and should, therefore, be used only where suitable precautions will be taken. In particular, rubber gloves and similar protective means are suitable precautions to be taken in order to be sure that the dye does not contact the skin. In actual practice, methylene blue is more satisfactory since it is safe. Also, of particular interest are acridine orange and Vistra green, the latter being a product of Amersham Pharmacia Biotech.

**[0010]** The mechanism of binding is not certain. As to ethidium bromide and acridine orange, it is believed that they enter the DNA molecule and are intercalated between the nucleic acid pairs of hydrogen bonds located where the halves of the molecule meet. On the other hand, methylene blue does not intercalate and is less tightly bound to the DNA molecule. As a result, it is easier to wash out when dipped in water.

**[0011]** In some cases, it is desirable to determine the effectiveness of the indicator and this is done by the use of spores of particular microorganisms which are placed therein. Spores of Bacillus subtilis and Bacillus stearothermophilus, standard test organisms, are quite resistant to heat. At the end of the cycle, the spores of the microorganisms are cultured in the usual way to determine whether there is any growth. No growth indicates that they have been killed and that the sterilization cycle was effective. If the culture grows, then the opposite conclusion is reached. These results are compared with those obtained from the test pack in which the DNA is located, in order to determine that the indicator is operating properly.

**[0012]** In a desirable form of the Invention, the DNA is first subjected to the sterilization cycle. After withdrawal from

the autoclave, it is deposited on the membrane (e.g. nitrocellulose or nylon), and a drop of dye solution is deposited thereon. Thereafter, it is dipped into water and observed. If the sterilization cycle is complete, the DNA will have been fragmented and/or the dye will not be bound thereto. Thus, when it is dipped into water, most (or all) of the dye will have been washed off. This is, of course, easily observable by the operator. It has been found particularly useful if some detergent is added to the wash water.

**[0013]** As a preferred form of the Invention, a further sample of the DNA is provided which is not introduced into the sterilization chamber. At the end of the cycle, this is dipped into the dye and then into the wash water and provides a standard for comparing the DNA that has undergone the sterilization cycle with the original. This aids in determining whether a change has occurred.

## DETAILED DESCRIPTION OF THE INVENTION

**[0014]** In the preferred form of the Invention, DNA in water solution is placed in a vial in the sterilizer. After the cycle is complete, the DNA is withdrawn from the sterilizer and dropped onto a suitable membrane such as nitrocellulose or nylon. This is followed by the application of the dye (e.g. methylene blue) on the DNA. At this point, the DNA is fully colored by the dye. It is then dipped into water and removed. If the sterilization cycle is complete, the DNA will have been fragmented and/or its ability to bind the dye will have been significantly reduced. As a result, most or all of the dye will wash off in the water and the membrane will evidence a substantial lightening of the color.

**[0015]** On the other hand, if the color does not lighten substantially, it indicates that the DNA remains substantially intact so that the dye remains bound to it. This indicates that the sterilization cycle is not complete and appropriate action must be taken. By including a small amount of the detergent in the water, the dye removal will be improved and the test will be more easily read.

**[0016]** The concentration of DNA in the DNA preparation should be 0.25 to 1.0 μg/μl as a practical and realistic range. Above the upper limit, there could be so much DNA present that, even with proper sterilization, there would be enough unbroken molecules to hold the dye so that the difference between the control (no breakdown) and the properly cycled DNA cannot be readily determined. On the other hand, if there is insufficient DNA (below the lower limit), then even an incomplete cycle will cause sufficient breakdown so that the remainder is insufficient to bind enough dye to maintain the color. Thus, outside the foregoing limits, the test becomes less reliable.

### Example 1

**[0017]** To test the effectiveness of the present Invention in gravity displacement sterilizers, standard DNA preparations (0.25 μg/μl in 300 μl total volume) were subjected to gravity displacement steam sterilization at 121°C (250 F) for various periods of time. The DNA containing vials were placed in Propper Bio-Challenge Packs along with Bi-OK Self-Contained Biological Indicators ($D_{250}$ = 1.8 minutes, 2.0 x $10^5$ spores per strip). One DNA containing vial and one biological indicator were in each pack and three such packs were used in each run.

**[0018]** The results obtained were as follows:

| Exposure Time | DNA Binding Affinity | Spore Growth (# survivors/ # exposed) |
|---|---|---|
| 0 min | ++++ | |
| 18 min | + | 5/6 |
| 30 min | - | 0/6 |

Spore growth was reported after 48 hours growth at 55°C.

**[0019]** In the Tables contained herein, each + represents 25% of the binding affinity, "-" indicates zero affinity.

### Example 2

**[0020]** Standard DNA preparations (0.25 μg/μl in 300 μl total volume) were subjected to prevacuum sterilization at 132°C (270 F) for various periods of time. The vials containing the DNA were placed in Propper Bi-OK Steam Packs along with Bi-OK Self Contained Biological Indicators ($D_{250}$ = 1.8 minutes, 2.0 x $10^5$ spores per strip). One vial containing DNA and one biological indicator were contained in each pack. Three packs were used for each run and the cycle consisted of four levels of vacuum (-26, -27, -27, and -27 inches of mercury, respectively), applied prior to steam input. The cycles were run for 1 minute, 2 minutes, 3 minutes, and 4 minutes.

**[0021]** The following results were obtained.

| Exposure Time | DNA Binding Affinity | Spore Growth (# survivors/ # exposed) |
|---|---|---|
| 0 min | ++++ | |
| 1 min | +++ | 5/6 |
| 2 min | ++ | 2/6 |
| 3 min | + | 0/6 |
| 4 min | - to +/- | 0/9 |

Spore growth was reported after 48 hours incubation at 55°C. The symbol "+/-" indicates a maximum of 10% binding affinity remaining.

**Example 3**

**[0022]** Standard DNA preparation (0.25 µg/µl and 0.5 µg/µl in 300 µl total volume) were subjected to 132 °C (270 F) for various periods of time. The preparation was placed within a Propper Bio-Challenge Pack and the prevacuum cycle consisted of three applications of vacuum (-26, -27, and -27 millimeters of mercury, respectively) drawn prior to steam input. The following binding affinities were noted.

| | DNA Concentration | | Spore Growth |
|---|---|---|---|
| Exposure Time | 25 µg/µl | 5 µg/µl | # Survivors / # Exposed |
| 1 min. | ++ | +++ | 6/6 |
| 2 min. | ++ | +++ | |
| 2.5 min. | + | +++ | 5/6 |
| 4 min. | - | ++ | 0/6 |

**[0023]** As the foregoing Examples amply demonstrate, the DNA indicator in accordance with the present Invention operates reliably for both prevacuum and gravity sterilizers.

**[0024]** Although only a limited number of specific embodiments have been expressly disclosed, the Invention is to be broadly construed and not to be limited except by the scope of the claims appended hereto.

**Claims**

1. A method of determining the effectiveness of a sterilization cycle, which includes application of heat and steam in a sterilization chamber, said method comprising

   placing a sterilization indicator in said sterilization chamber prior to beginning said sterilization cycle, said sterilization indicator including a DNA preparation containing from 0.25 to 1.0 µg of initial DNA per µl of water, said initial DNA being able to be fragmented on being subjected to said sterilization cycle to form DNA that has undergone said sterilization cycle,
   carrying out said sterilization cycle whereby at least some of said initial DNA is converted into said DNA that has undergone said sterilization cycle,
   withdrawing said DNA that has undergone said sterilization cycle from said chamber, contacting said DNA that has undergone said sterilization cycle with a dye capable of being bound to said initial DNA and not by fragmented DNA, dipping said DNA that has undergone said sterilization cycle containing said dye into wash water, and thereafter observing said DNA that has undergone said sterilization cycle.

2. The method of Claim 1 wherein said initial DNA is on a support.

3. The method of Claim 2 wherein said support is a plastic having a negative surface potential.

4. The method of Claim 2 wherein said support is a membrane.

5. The method of Claim 4 wherein said membrane is non-porous.

6. The method of Claim 2 or 4 wherein said support is of nitrocellulose or nylon.

7. The method of any one of Claims 1 to 6 wherein said DNA is double stranded.

8. The method of any one of Claims 1 to 7 wherein said initial DNA is high molecular weight.

9. The method of Claim 1 wherein said wash water contains a detergent.

10. The method of any one of Claims 1 to 9 wherein said sterilization indicator comprises a test micro-organism, culturing said micro-organism after removal from said sterilization chamber whereby the effectiveness of said cycle and said indicator can be determined.

11. The method of Claim 10 wherein said micro-organism is selected from the group consisting of Bacillus subtilis and Bacillus stearothemophilus.

12. The method of any one of Claims 1 to 11 wherein said dye is selected from the group consisting of ethidium bromide, methylene blue, acridine orange, and Vistra green.

13. The method of Claim 12 wherein said dye is methylene blue.

14. The method of Claim 2 wherein said support is a porous paper inserted with said initial DNA.

15. The method of any one of Claims 1 to 13 wherein said initial DNA is provided on a substrate which is not introduced into said chamber, dipping said substrate into said solution of said dye and then into said second solvent, thereby acting as a standard of comparison for said DNA that has undergone said sterilization cycle.

16. The method of any one of Claims 1 to 15 wherein said sterilization cycle is of the prevacuum type.

17. The method of any one of Claims 1 to 16 wherein said sterilization cycle is of the gravity type.

18. The method of any one of Claims 1 to 17 wherein said DNA preparation contains 0.25 to 0.50 μg of initial DNA per μl of water.

**Patentansprüche**

1. Verfahren zur Bestimmung der Wirksamkeit eines Sterilisierungszyklus, das die Anwendung von Wärme und Dampf in einer Sterilisationskammer einschliesst, das Verfahren umfasst:

   Plazieren eines Sterilisationsindikators in die Sterilisationskammer vor dem Beginn des Sterilisationszyklus, der Sterilisationsindikator schliesst eine DNA-Zubereitung, die 0,25-1,0 μg anfänglicher DNA pro μl Wasser enthält, ein, die anfängliche DNA ist in der Lage, indem sie dem Sterilisationszyklus unterworfen wird, fragmentiert zu werden, wodurch DNA gebildet wird, die den Sterilisationszyklus durchlaufen hat,

   Durchführung des Sterilisationszyklus, wodurch zumindest ein Teil der anfänglichen DNA in die DNA, die den Sterilisationszyklus durchlaufen hat, umgewandelt wird,

   Entnahme der DNA, die den Sterilisationszyklus durchlaufen hat, aus der Kammer, Kontaktieren der DNA, die den Sterilisationszyklus durchlaufen hat, mit einem Farbstoff, der in der Lage ist, an die anfängliche DNA, aber nicht durch die fragmentierte DNA gebunden zu werden, Eintauchen der DNA, die den Sterilisationszyklus durchlaufen hat und den Farbstoff enthält, in Waschwasser, und anschliessende Beobachtung der DNA, die den Sterilisationszyklus durchlaufen hat.

2. Verfahren gemäss Anspruch 1, worin die anfängliche DNA auf einem Träger befindlich ist.

3. Verfahren gemäss Anspruch 2, worin der Träger ein Kunststoff mit einem negativen Oberflächenpotential ist.

4. Verfahren gemäss Anspruch 2, worin der Träger eine Membran ist.

5. Verfahren gemäss Anspruch 4, worin die Membran nichtporös ist.

6. Verfahren gemäss Anspruch 2 oder 4, worin der Träger aus Nitrocellulose oder Nylon besteht.

7. Verfahren gemäss mindestens einem der Ansprüche 1 bis 6, worin die DNA doppelsträngig ist.

8. Verfahren gemäss mindestens einem der Ansprüche 1 bis 7, worin die anfängliche DNA ein hohes Molekulargewicht besitzt.

9. Verfahren gemäss Anspruch 1, worin das Waschwasser ein Reinigungsmittel enthält.

10. Verfahren gemäss mindestens einem der Ansprüche 1 bis 9, worin der Sterilisationsindikator einen Testmikroorganismus umfasst und die Effektivität des Zyklus und des Indikators bestimmt werden kann durch Kultivierung des Mikroorganismus nach Entnahme aus der Sterilisationskammer.

11. Verfahren gemäss Anspruch 10, worin der Mikroorganismus ausgewählt ist aus Bacillus subtilis und Bacillus stearothemophilus.

12. Verfahren gemäss mindestens einem der Ansprüche 1 bis 11, worin der Farbstoff ausgewählt ist aus Ethidiumbromid, Methylenblau, Acridinorange und Vistragrün.

13. Verfahren gemäss Anspruch 12, worin der Farbstoff Methylenblau ist.

14. Verfahren gemäss Anspruch 2, worin der Träger ein poröses Papier ist, in das die anfängliche DNA insertiert ist.

15. Verfahren gemäss mindestens einem der Ansprüche 1 bis 13, worin die anfängliche DNA auf einem Substrat bereitgestellt wird, das nicht in die Kammer eingeführt wird, und, durch Eintauchen des Substrats in die Lösung des Farbstoffs und anschliessend in das zweite Lösungsmittel, als Standard zum Vergleich für die DNA, die den Sterilisationszyklus durchlaufen hat, dient.

16. Verfahren gemäss mindestens einem der Ansprüche 1 bis 15, worin der Sterilisationszyklus vom Vorvakuumtyp ist.

17. Verfahren gemäss mindestens einem der Ansprüche 1 bis 16, worin der Sterilisationszyklus vom Gravitationstyp ist.

18. Verfahren gemäss mindestens einem der Ansprüche 1 bis 17, worin die DNA-Zubereitung 0,25-0,50 µg anfänglicher DNA pro µl Wasser enthält.

**Revendications**

1. Procédé de détermination de l'efficacité d'un cycle de stérilisation qui inclut l'application de chaleur et de vapeur dans une chambre de stérilisation, ledit procédé comprenant

   la mise en place d'un indicateur de stérilisation dans ladite chambre de stérilisation avant le début dudit cycle de stérilisation, ledit indicateur de stérilisation incluant une préparation d'ADN contenant de 0,25 à 1,0 µg d'ADN initial par µl d'eau, ledit ADN initial étant capable d'être fragmenté quand il est soumis audit cycle de stérilisation pour former un ADN qui a subi ledit cycle de stérilisation,
   la mise en oeuvre dudit cycle de stérilisation de sorte qu'au moins une certaine partie dudit ADN initial est convertie en ledit ADN qui a subi ledit cycle de stérilisation,
   le retrait dudit ADN qui a subi ledit cycle de stérilisation de ladite chambre, la mise en contact dudit ADN qui a subi ledit cycle de stérilisation avec un colorant capable d'être lié audit ADN initial et non par de l'ADN fragmenté, l'immersion dudit ADN qui a subi ledit cycle de stérilisation contenant ledit colorant dans de l'eau de lavage, puis l'observation dudit ADN qui a subi ledit cycle de stérilisation.

2. Procédé selon la revendication 1 où ledit ADN initial est sur un support.

3. Procédé selon la revendication 2 où ledit support est une matière plastique ayant un potentiel de surface négatif.

4. Procédé selon la revendication 2 où ledit support est une membrane.

5. Procédé selon la revendication 4 où ladite membrane est non-poreuse.

6. Procédé selon la revendication 2 ou 4 où ledit support est en nitrocellulose ou en nylon.

7. Procédé selon l'une quelconque des revendications 1 à 6 où ledit ADN est double brin.

8. Procédé selon l'une quelconque des revendications 1 à 7 où ledit ADN initial est à haute masse moléculaire.

9. Procédé selon la revendication 1 où ladite eau de lavage contient un détergent.

10. Procédé selon l'une quelconque des revendications 1 à 9 où ledit indicateur de stérilisation comprend un micro-organisme test, la culture dudit micro-organisme après le retrait de ladite chambre de stérilisation de sorte que l'efficacité dudit cycle et dudit indicateur peut être déterminée.

11. Procédé selon la revendication 10 où ledit micro-organisme est choisi dans le groupe consistant en Bacillus subtilis et Bacillus stearothermophilus.

12. Procédé selon l'une quelconque des revendications 1 à 11 où ledit colorant est choisi dans le groupe consistant en le bromure d'éthidium, le bleu de méthylène, l'orange d'acridine et le vert Vistra.

13. Procédé selon la revendication 12 où ledit colorant est le bleu de méthylène.

14. Procédé selon la revendication 2 où ledit support est un papier poreux inséré avec ledit ADN initial.

15. Procédé selon l'une quelconque des revendications 1 à 13 où ledit ADN initial est fourni sur un substrat qui n'est pas introduit dans ladite chambre, l'immersion dudit substrat dans ladite solution dudit colorant puis dans ledit second solvant, pour jouer le rôle d'étalon de comparaison pour ledit ADN qui a subi ledit cycle de stérilisation.

16. Procédé selon l'une quelconque des revendications 1 à 15 où ledit cycle de stérilisation est du type à vide préalable.

17. Procédé selon l'une quelconque des revendications 1 à 16 où ledit cycle de stérilisation est du type à gravité.

18. Procédé selon l'une quelconque des revendications 1 à 17 où ladite préparation d'ADN contient 0,25 à 0,50 µg d'ADN initial par µl d'eau.